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COMMUNICATION

Lipase-embedded silica nanoparticles with oil-filled core–shell structure: stable and recyclable platforms for biocatalysts†

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Lipase enzyme was embedded within silica nanoparticles with oil-filled core–shell structure. The enzyme embedded within such architecture retained all of its activity and showed high catalytic performance both in water and in organic media with optimal stability and recyclability.

Biocatalysts are highly selective entities, but by nature show limited stability and recyclability. Robust immobilization that preserves the activity of biocatalysts has been a technique of great importance for a range of applications in biotechnology including food processing, enzymatic catalysis, drug-delivery systems and biosensors.[1] A common strategy for immobilizing enzymes is their encapsulation inside sol–gel derived silica matrices, which function to efficiently protect the encapsulated enzymes from the surrounding environment.[2–6] However, due to nonporous structure, most studies showed lower specific activity than that of the free enzymes. Silica hollow spheres have been also envisaged as promising carriers for biomolecules because the void volume can be used for encapsulation of various guests and the silica shell acts as a physical barrier to protect them from the surrounding environment.[7] However, the synthetic methodology requires intricate multiple preparation steps and careful control of the synthetic conditions to circumvent the denaturation of enzymes. Although chemical/physical fixation within the pore channels of functionalized mesoporous silica[8–12] or onto the void cavities of silica-based macrocellular foam[13] has been widely used because of their rigid and uniform open-pore structure, the complicated synthetic procedures and significant leaching during their use limit their applicability. A worthwhile challenge to this issue is to develop a facile and scalable immobilization approach while preserving catalytic performance of enzymes.

In this communication, we present a novel protocol for immobilizing enzymes retaining all of their activity and increasing the stability and recyclability: enzymes are directly entrapped within spherical silica nanoparticles having oil-filled core and oil-induced mesoporous silica shell. The advantages of this synthetic route are the ease of fabrication, ambient synthetic conditions and unique architecture including oil in its core. We selected *Candida Antarctica Lipase A* (CALA) for this work because it has been widely investigated as a promising biocatalyst for transesterification reaction involved in biodiesel production from vegetable oils.[14] The CALA-embedded oil-filled silica nanoparticles (CALA@OSN) were synthesized by an oil-in-water emulsion templating method,[15] which was carried out by adding enzymes (30 mg per g SiO_2) into the emulsion consisting of oleic acid (OA) and water (pH 5.3). After addition of silicon sources (TEOS and APTES), the resulting solution was aged for 24 h at 50 °C, during which the pH value was 9.6 at the maximum and finally approached the neutral value of 7.3 (Fig. S1 in ESI†).

Fig. 1 summarizes the results of structural analyses of CALA@OSN. The SEM image of CALA@OSN shows monodispersed spherical silica nanoparticles with an average particle size of 246 nm (Fig. 1(b)). XRD and N_2 adsorption measurements identified nonporous structure of as-synthesized CALA@OSN, but confirmed OA-induced mesoporous structure of the calcined sample. The calcined sample exhibits a broad

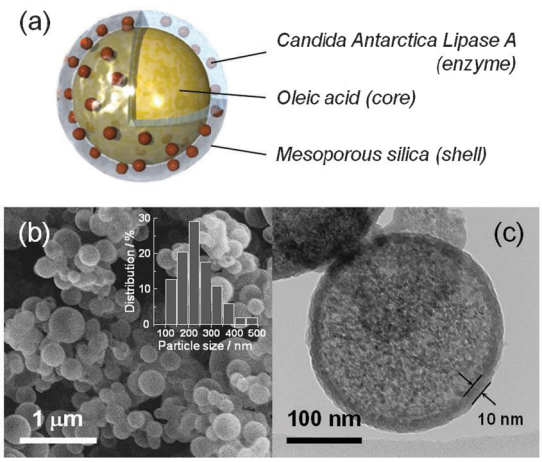

Fig. 1 (a) Illustration of structure, (b) SEM micrograph (inset shows a particle size distribution diagram) and (c) TEM micrograph of CALA@OSN.

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† Electronic supplementary information (ESI) available: Detailed information on the material synthesis, XRD and sorption data for CALA@OSN and CALA@MCM-41 and activity characterization results. See DOI: 10.1039/c2cc17896f

diffraction peak associated with 1-D wormhole-like mesopores at 1.23° and a distinct pore distribution with an average pore diameter of 4.2 nm (Fig. S2 in ESI†). The specific surface area and total pore volume were determined to be 26.2 $m^2\ g^{-1}$ and 0.284 $cm^3\ g^{-1}$ for an as-synthesized sample and 325 $m^2\ g^{-1}$ and 0.472 $cm^3\ g^{-1}$ for a calcined sample. While such meso-porosity in the silica shell is not observed in the TEM image because of disordered arrangement of the pores (Fig. 1(c)), these observations evidence that the CALA@OSN consists of the core of OA and the mesoporous silica shell filled with OA as illustrated in Fig. 1(a).

In order to disclose the whereabouts of the incorporated enzymes, bovine serum albumin–fluorescein isothiocyanate conjugate (BSAf), whose molecular dimensions (7.0 × 7.0 × 5.0 nm)[8a–c] are close to those of CALA (6.3 × 5.6 × 4.2 nm),[16] was used as a guest molecule instead of CALA. The suspension of BSAf-embedded silica nanoparticles is separated into a yellow precipitate and a clear supernatant by centrifugation (Fig. 2(a)). The remaining enzyme amount in the supernatant and wash fractions was negligible, suggesting that most of the soluble biomolecules were incorporated within the composite materials. Furthermore, the fluorescence micrograph unambiguously confirms that the biomolecules are mostly sequestered within the silica shell, not in the oil phase (Fig. 2(b)). These results indicate that our enzyme immobilization protocol spontaneously leads to the incorporation of the enzymes within the silica matrix of the shell during silica network formation.

We examined the activity of CALA@OSN by hydrolysis of *n*-butylacetate in phosphorous buffer (pH 7.0) as a test reaction and compared with that of free enzyme and CALA immobilized

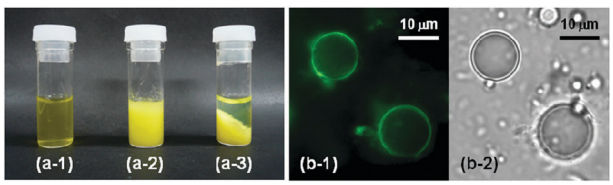

Fig. 2 (a-1) An aqueous solution of bovine serum albumin–fluorescein isothiocyanate conjugate (BSAf) and suspensions of BSAf@OSN (a-2) before and (a-3) after centrifugation. (b-1) Fluorescence microscopy image and (b-2) optical microscopy image of BSAf@OSN with huge particle size taken using an Olympus confocal system.

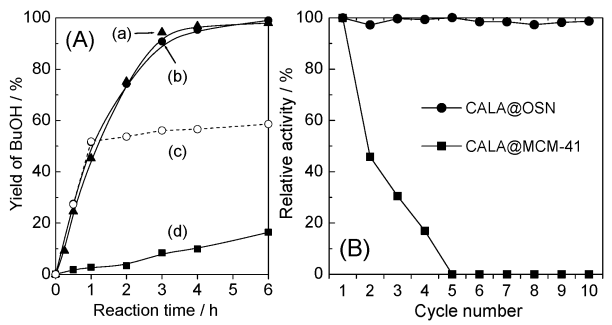

Fig. 3 (A) Hydrolysis of *n*-butylacetate in Na-phosphate buffer (pH 7.0) catalyzed by (a) free CALA, (b) CALA@OSN, (c) after removal of CALA@OSN by filtration of the reaction mixture (the reaction had previously been allowed to proceed for 1 h) and (d) CALA@MCM-41. (B) Reproducibility test. *Reaction conditions*: catalyst including 1.0 mg of CALA, *n*-butylacetate aqueous solution (50 mM, 1 mL), Na-phosphate buffer (50 mM, 9 mL), 40 °C.

on MCM-41 (CALA@MCM-41: for N_2 adsorption data, see Fig. S3 and Table S1 in ESI†). As shown in Fig. 3(A), CALA@OSN exhibits as high a reaction rate as that of free enzyme, indicating negligible loss of enzymatic activity upon immobilization. Furthermore, the reaction was quenched after recovery of the silica particles from the reaction media by simple filtration, whereas free enzyme is unrecoverable due to its homogeneity. More significantly, the catalytic efficiency is at least up to 10 times reproduced for CALA@OSN while retaining more than 97% of the initial activity in each cycle (Fig. 3(B)), demonstrating its excellent recyclability. The retention of enzymatic activity and fine reproducibility can be attributed to the mild synthetic conditions (ambient temperature and narrow pH range) and the robust immobilization ability of the silica matrix without affecting the active sites and disruption of subunits, respectively. In comparison, CALA@MCM-41 obtained by conventional wet-impregnation showed a quite poor reaction rate (16.4% conversion after 6 h) and significant decrease in enzymatic activity during repeated use due to enzyme denaturation and substantial leaching to the reaction medium. One of the critical disadvantages of the conventional immobilization on silicate materials is the low catalytic efficiency of enzymes mainly derived from nonporosity of the silica-gel or close packing within the mesopore channels, thereby resulting in a lower activity than that of free enzymes. Contrarily to these approaches, this synthetic route enables us to achieve as high a catalytic efficiency as that of free enzyme. This is presumably because of high accessibility between enzymes and reactants; the thickness of the silica shell determined from the TEM image is *ca.* 10 nm (Fig. 1(c)), which is slightly larger than the molecular dimensions of CALA, indicating that CALA molecules are being embedded in the vicinity of the outer surface. It is deduced that such a configuration allows reactants to efficiently access to the active sites of enzymes.

It is worth mentioning that CALA@OSN can be used as an efficient heterogeneous biocatalyst in the hydrolysis and transesterification reactions of triglycerides using *n*-heptane as an organic solvent owing to its amphiphilicity. As shown in Fig. 4, CALA@OSN retains most of its activity in multiple catalytic cycles in both catalytic reactions, whereas CALA@MCM-41 loses its whole activity after a few cycles (for detailed catalytic results, see Fig. S4 and S5 and Table S1 in ESI†), suggesting that triglycerides in the organic medium have easy access to the active sites of the embedded CALA. It is believed that the OA-induced organophilicity of OSN increases the affinity with the organic media and leads to a prominent catalytic activity,[17] because enzyme itself is insoluble in the organic media. The attainment of high catalytic performance in organic media using enzyme-based heterogeneous biocatalysts is currently the research area of great industrial importance, because most of pharmaceutical or biofuel end products are insoluble in water.[13] The above results, therefore, suggest the availability of this material to be used as an industrially practicable heterogeneous biocatalyst.

To evaluate the suitability of OSN as stable platforms for enzymes, we also examined the enzymatic activity as a function of solution pH and temperature. As seen in Fig. 5, the CALA@OSN exhibits a higher enzymatic activity than that of free enzyme over a wide pH range. The free enzyme in solution is readily denatured

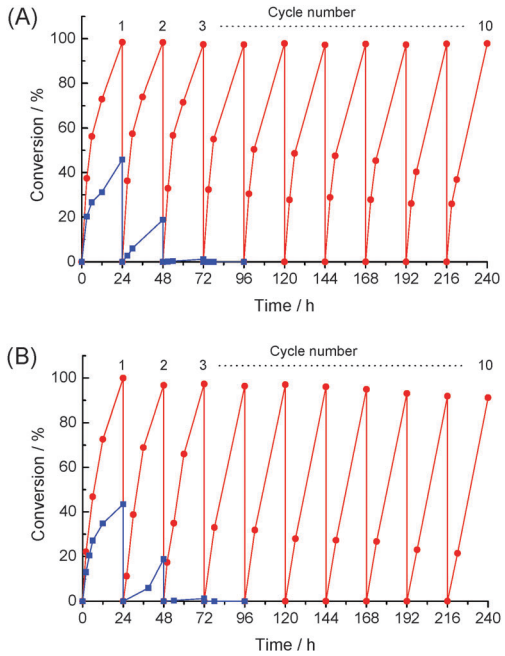

Fig. 4 Comparison of recyclability of (●) CALA@OSN and (■) CALA@MCM-41 in (A) hydrolysis of tricaprylin ($C_{27}H_{50}O_6$) in water-saturated *n*-heptane and (B) transesterification of tricaprylin with ethanol in *n*-heptane. *Reaction conditions*: catalyst containing 4.0 mg of CALA, tricaprylin (10 μmol), either water-saturated *n*-heptane or 120 μmol of ethanol-containing *n*-heptane (10 mL), 40 °C.

during the reaction at 60–80 °C (51–83% decrease in relative activity). In contrast, CALA@OSN retains most of its activity under the same conditions, proving enhanced thermostability for this composite material. This is attributed to the stabilizing effect of the silica matrix, which prevents extensive conformational changes typical of thermal denaturation.[18] The ability to retain enzyme activity in wide pH ranges and at high temperatures provides a number of processing advantages such as improved reaction rate and substrate solubility, thereby expanding the range of applications of enzymes.

In summary, we fabricated a new type of heterogeneous biocatalyst by integrating multi-components, *i.e.*, enzymes, silica shell and oil core, into isolated spherical silica nanoparticles, and

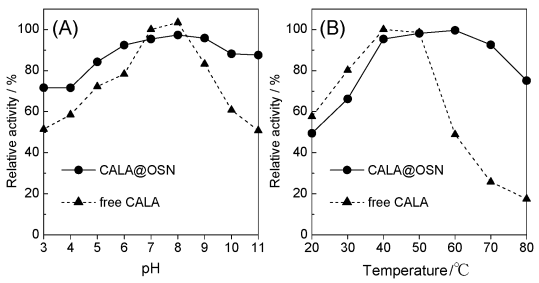

Fig. 5 Enzymatic activity of (●) CALA@OSN and (▲) native CALA at different (A) pH and (B) temperature. Relative activity was assessed by hydrolysis of *n*-butylacetate, which was performed in the mixture of 1 mL of *n*-butylacetate aqueous solution (50 mM), 9 mL of 50 mM buffer solution and the catalyst including 1.0 mg of CALA. The activities were normalized by that of free CALA at pH 7.0 and at 40 °C.

demonstrated that the enzyme embedded within such architecture retained all of its activity and showed high catalytic performance both in water and in organic media with increased stability and recyclability. The enzyme immobilization protocol presented in this study provides several advantages: mild synthetic conditions that preserve the integrity of enzymes, low cost and convenience in preparation, efficient recovery and recyclability of the catalysts, amphiphilicity induced by oil-filled core–shell structure and versatility in choice of enzymes. With those favourable characteristics, this method will be a promising approach for synthesizing highly stable and readily recoverable biocatalysts.

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